Available online at www.sciencedirect.com
ScienceDirect
South African Journal of Botany 76 (2010) 717–725
SOUTH AFRICAN JOURNAL OF BOTANY
www.elsevier.com/locate/sajb

Scent chemistry and patterns of thermogenesis in male and female cones of the African cycad *Encephalartos natalensis* (Zamiaceae)

T.N. Suinyuy [a,d,c,*], J.S. Donaldson [a,b,d], S.D. Johnson [c]

[a] *KRC, SA National Biodiversity Institute, Private Bag X7, Claremont 7735, Cape Town, South Africa*
[b] *Research Associate, Fairchild Tropical Botanic Garden, Miami, FL, USA*
[c] *School of Biological and Conservation Sciences, University of KwaZulu-Natal Pietermaritzburg, Private Bag X01, Scottsville 3201, South Africa*
[d] *Department of Botany, University of Cape Town, South Africa*

Received 20 June 2010; received in revised form 11 August 2010; accepted 19 August 2010

Abstract

Most, if not all, extant cycads are pollinated by insects which use the cones as larval brood sites. These interactions appear to be mediated by cone volatiles, and, in some species, by patterns of thermogenesis. We investigated the chemical composition of volatile emissions and patterns of thermogenesis in cones of the South African cycad *Encephalartos natalensis,* using a gas chromatograph–mass spectrometer (GC–MS) and miniature temperature data loggers (ibuttons), respectively. This was done during various developmental stages (before and during receptivity and pollen release) for both female and male cones. A total of 31 compounds were identified in headspace samples; 17 of which were common to both sexes, 12 found only in male cones, and two found only in female cones. The major volatiles in pollen and female cones are (3*E*)-1,3-octadiene (averaging 54.25% and 15.82% of total emissions), (3*E*,5*Z*)-1,3,5-octatriene (averaging 13.37% and 47.66%), and α-pinene (averaging 16.29% and 12.24%). Female cones were not thermogenic before and during receptivity whereas pollen cones were thermogenic during pollen shedding. Thermogenesis of male cones occurred between 1400 h and 1530 h on successive afternoons, reaching an average of c. 10.5 °C above ambient temperature. Volatile emissions and thermogenesis occurred in association with insect activity on the cones suggesting that they both play a role in regulating insect behaviour.

Keywords: African cycads; Gas chromatography–mass spectrometry; Insect visitors; KwaZulu-Natal; Pollination; Thermogenesis; Volatile emissions; Zamiaceae

1. Introduction

Several highly specialized plant–pollinator interactions have been shown to be mediated by emission of volatiles in conjunction with heat production (Bronstein et al., 2006; Jürgens, 2009; Pellmyr and Thien, 1986; Pellmyr, 1992; Seymour and Schultze-Motel, 1997). Although this area of research has focused mostly on flowering plants, there is an emerging body of work on cycads, which shows that volatile emissions and thermogenesis also influence pollinator behaviour in these early seed plants (Donaldson, 1997; Seymour et al., 2004; Tang, 1987a,b; Terry et al., 2004, 2007). Studies of cycads can provide important insights into the evolution of plant–pollinator interactions. Firstly, cycads are the oldest group of extant seed plants and the evolution of insect pollination mutualisms in cycads may have pre-dated the evolution of insect pollination in angiosperms (Norstog and Nicholls, 1997; Stevenson et al., 1998). Secondly, it has been hypothesized that plant volatiles originated as herbivore deterrents and later served a function as attractants (Pellmyr and Thien, 1986). All known cycad–pollinator interactions involve insect herbivores that feed on cycad reproductive structures, and this means that cycads are a good model to test this hypothesis. Finally, studies of cycad–insect associations indicate that some cycad–pollinator interactions could have originated through a shift from angiosperm host plants to cycads (Oberprieler, 2004). Therefore, studies of cycads may contribute to a better understanding of the evolution and function of volatile compounds in herbivore and pollinator attraction, and the occurrence of host shifts.

* Corresponding author. KRC, SA National Biodiversity Institute, Private Bag X7, Claremont 7735, Cape Town, South Africa.
E-mail address: T.Suinyuy@sanbi.org.za (T.N. Suinyuy).

0254-6299/$ - see front matter © 2010 SAAB. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.sajb.2010.08.014

Early observations of African cycads indicated that male cones emitted volatiles and produced heat (thermogenesis) during pollen shed, and that insects visited male and female cones at the time of odour emission and heat production (Jacot-Guillarmod, 1958; Pearson, 1906; Rattray, 1913; Tang, 1987a). However, the role of insects and the function of volatile emissions and thermogenesis in cycad pollination went untested for a long time because of the prevailing paradigm that all cycads were wind-pollinated (e.g. Chamberlain, 1935). Subsequent studies have shown that at least four African cycad species, *Encephalartos cycadifolius*, *E. villosus*, *E. friderici-guilielmi* and *Stangeria eriopus,* are pollinated by beetles (Donaldson et al., 1995; Donaldson, 1997; Proches and Johnson, 2009; Suinyuy et al., 2009). These studies, together with those of other extant species (e.g. *Zamia furfuracea*, Norstog et al., 1986; *Z. pumila*, Tang, 1987b; *Macrozamia communis*, Terry, 2001; *Bowenia*, Wilson, 2002; *Lepidozamia peroffskyana*, Hall et al., 2004; *M. machinii* and *M. lucida*, Terry et al., 2005; and *Cycas*, Kono and Tobe, 2007) provide experimental evidence for insect pollination in seven cycad genera and strong circumstantial evidence for insect pollination in the remaining three genera, *Ceratozamia* and *Dioon* (Vovides, 1991), and *Microcycas* (Vovides et al., 1997). Wind pollination, if it occurs, is likely to be an exception to this general pattern of insect pollination, or may occur in conjunction with insect pollination (Niklas and Norstog, 1984). Although insect pollination is apparently common in extant cycads, the role of cone volatiles and thermogenesis in mediating cycad–insect interactions is still poorly known.

Early studies of other African *Encephalartos* cycads have identified thermogenesis in male cones of *E. altensteinii* and *E. lehmannii* (Jacot-Guillarmod, 1958) and in male and/or female cones of *E. barteri*, *E. bubalinus*, *E. ferox*, *E. gratus*, *E. hildebrandtii*, *E. longifolius*, and *E. manikensis* (Tang, 1987a). Gas chromatography–mass spectrometry (GC–MS) analyses of volatiles have been conducted only for *E. altensteinii* and *E. villosus* (Pellmyr et al., 1991; Suinyuy et al., in press). Therefore, further studies are required to determine the possible role that cycad cone volatile and thermogenesis plays in regulating insect behaviour in *Encephalartos*.

In this study, we examined cone volatiles, and thermogenesis, as well as insect visitors, in *Encephalartos natalensis* Dyer and Verdoorn, a cycad in which both female and male cones are known to be visited by a variety of insect species (Oberprieler, 1995; Vorster, 1995). In our study, we measured thermogenesis, analysed volatile compounds, and surveyed insect visitors in both male and female cones at different stages of development (before and during pollen shedding and receptivity). We compare the results from these studies to the available data for other cycads and discuss the possible function of cone volatiles and thermogenesis for pollination.

2. Materials and methods

2.1. Study species and system

E. natalensis, commonly called the Natal cycad, is endemic to South Africa and is widely distributed in KwaZulu-Natal. The species occurs mostly inland and is often associated with rocky outcrops, cliffs and escarpments in hilly terrain (Jones, 1993). The climate in this area is characterised by hot and wet summers with cold and dry winters and occasional frost. Different forms exist that are differentiated mostly by leaf characteristics and the extent of woolly tomentum in the crown (Giddy, 1978; Goode, 2001). We sampled *E. natalensis* specimens of unknown origin at the Pietermaritzburg campus of the University of KwaZulu-Natal (UKZN), as well as plants occurring naturally at the KwaZulu-Natal National Botanical Garden, Pietermaritzburg, and the conservation area of Hilton College just north of Pietermaritzburg. Mature specimens have an erect or reclining stem and older plants can be between 4 and 6 m tall (Fig. 1a). Female and male plants produce between one and five cones per stem. Female cones are 50 to 60 cm long and 25 to 30 cm in diameter while male cones are 45 to 40 cm long and 10 to 12 cm in diameter (Fig. 1a,b). Female and male cones have a woolly tomentum early in their development but gradually lose their woolliness as they mature (Giddy, 1978). Male cones are pale yellow when mature and the cone extends so that the sporophylls separate and pollen is freely dispersed from the cone (Tang, 1987a).

2.2. Sampling of volatile compounds

Headspace sampling was used to collect volatiles from male and female cones before and during pollen release and receptivity, respectively. Polyacetate bags (Nalo Bratfolie Kalle GmbH-Germany) were placed over the cone just prior to sampling in

Fig. 1. (a) Female *Encephalartos natalensis* with receptive cones; (b) male *Encephalartos natalensis* with male cones at pollen shed stage.

order to concentrate the volatile compounds. Air from inside the bags was suctioned into a chromatoprobe trap containing 2 mg of a 50:50 mixture of Tenax TA® (Alltech Associates, USA) and activated charcoal (Carbotrap™, Supelco, USA) using a portable field battery operated pump (Spectrex Personal Air Sampler PAS 500, USA) calibrated at 200 ml/min. Cone volatiles were collected in the afternoon (between 1330 h and 1630 h) before and at pollen shed stage for male cones, and before and at receptive stage (gaps present between upper sporophylls) for female cones. Air samples were simultaneously collected from empty polyacetate bags as controls to identify background contamination. The adsorbent Tenax TA used in the study is commonly used to trap volatile compounds and has a high thermal stability up to 350 °C, which allows for thermal desorption in GC analysis (Tholl and Röse, 2006).

Volatile samples were analysed using a coupled Varian 3800 gas chromatograph (Varian Palo Alto, California, USA) and Varian 1200 mass spectrometer (GC–MS). The GC was equipped with a Carbowax column (DB-wax) of 30 m × 0.32 mm internal diameter x 0.25 μm film thickness (Alltech, Deerfield, Illinois, USA). Helium was used as the carrier gas at a flow rate of 1 mL/min. Traps containing the adsorbent and volatiles were placed in a Varian 1079 injector by means of a 'Chromatoprobe' fitting and thermally desorbed. After a 3 min hold at 40 °C, the GC oven was ramped up to 240 °C at 10 °C/min and held there for 12 min. Compound identification was carried out using the NIST05 mass spectral library and comparisons with retention times of authentic standards, where available, as well as comparisons between calculated Kovats retention indices and those published in the literature. A homologous series of alkanes (C8–C20) was used to determine Kovats retention indices. All reference compounds used for retention time comparisons were obtained from Sigma Aldrich Inc. GmbH, Germany, except (3*E*)-1,3-octadiene which was obtained from ChemSampco, USA. Compounds present at higher or similar percentages in controls were considered as contaminants and excluded from the analysis.

2.3. Thermogenesis

Temperatures of male and female cones of *E. natalensis* were monitored *in situ* for thermogenesis events before and during pollen release and receptivity, respectively. Temperature was measured using ibuttons (Fairbank technology USA). An ibutton was inserted between the cone sporophylls to measure cone temperature and a second ibutton was placed in between the leaves of the same cycad to measure ambient temperature. Both ibuttons were positioned to avoid direct exposure to sunlight. The ibuttons were set to record the temperature at 10 minute intervals for the duration of the pollination period of the cones.

2.4. Insect visitors to male and female cones of E. natalensis

To determine which insects visited *E. natalensis*, female and male cones were sampled before and during receptivity and pollen shedding respectively. Cones on cycads growing naturally at Hilton College Conservation area and the forest areas of KZN National Botanical Garden were examined around the same time that volatiles were sampled (1300 h–1530 h). To survey insects from male cones, the cone was placed over a beating sheet and tapped to dislodge all the insects onto the sheet. Seven male cones were surveyed and all the insects were counted, recorded and stored in 70% ethanol. Only insects crawling on the surface of female cones were sampled as these could be collected and stored in alcohol without damaging the female cone.

2.5. Statistical analysis

We used the Primer 6 programme (Clarke and Gorley, 2006) to analyse and compare scent profiles from female and male cones before and during receptivity and pollen shedding. Non-metric multidimensional scaling (NMDS), based on Bray–Curtis similarities of square root transformed data, was used to detect similarities among samples. To evaluate how well or poorly the particular configuration reproduces the observed distance matrix, the stress value is given. The smaller the stress value, the better the fit of the reproduced ordination to the observed distance matrix (Clarke, 1993). Differences in scent profiles between stages and sex was assessed by ANOSIM (Clarke and Gorley, 2006) with 10 000 random permutations.

3. Results

3.1. Volatile emission

The volatiles emitted by female and male cones of *E. natalensis* before and during receptivity and pollen release are summarised in Table 1. The compounds are identified by common names and CAS (Chemical Abstract Service) registry numbers and listed according to estimated Kovats retention index (KRI). In total, 31 compounds were found, which included 13 fatty acid derivatives (four unsaturated hydrocarbons, four aldehydes, four ketones, and one alcohol), seven benzenoids, ten terpenoids (nine monoterpenes and one sesquiterpene), and one nitrogen-containing compound (Table 1). In general, there were fewer compounds from female cones, with 20 of the 31 compounds recorded from pre-receptive or receptive cones. In contrast, 29 of the 31 compounds were found in male cone samples taken either before or during pollen shed. Analysis of odour components showed that most volatile compounds were specific to particular cone stages, while others were sex specific, or even sex and cone stage specific. Before pollen release and receptivity, the major compounds, in male and female cones respectively, were benzaldehyde (50.3 and 63.1%), heptanal (35.2 and 12.8%) and linalool (3.1 and 10.4%) (Table 1). The most frequently occurring compounds in cones before pollen shed and receptivity were benzaldehyde and heptanal (found in all seven pre-pollen shedding and all eight pre-receptivity cones), 1-octen-3-ol (in six male cones), phenol (six male and eight female cones), linalool (five male and seven female cones) and benzyl alcohol and dihydro-2(3)-furanone (in all eight female cones). Two compounds were frequently emitted in relatively small amounts in both male and female cones, respectively: 1-octen-3-ol (4.2 and 0.3%) and phenol, (3.5 and 2.0%). The emission of dihydro-2(3)-furanone by the female

Table 1
Occurrence and relative amounts of volatile compounds emitted by male and female cones of *E. natalensis* before and during pollen shedding and receptivity, respectively. Compounds are identified by common names and CAS (Chemical Abstracts Service) registry number, and listed according to estimated Kovats retention index (KRI) within each compound class. Values are mean (±S.E) percentages of the total peak area. The number of samples in which the compound was identified is given in parentheses.

Compound	CAS	KRI	Male cones		Female cones	
			Pre-pollen shed	Pollen shed	Pre-receptive	Receptivity
			n=7	*n*=16	*n*=8	*n*=11
Aliphatics						
Unsaturated hydrocarbons						
(3*E*)-1,3-Octadiene [a]	1002-33-1	1062	–	54.25±10.27 (11)	–	15.82±9.60 (7)
(3*E*,5*Z*)-1,3,5-Octatriene [b]	40087-61-4	1148	–	13.37±4.60 (11)	–	47.66±13.21 (8)
(*E*,*E*,*E*)-2,4,6-Octatriene [b]	15192-80-0	1198	–	0.25±0.18 (6)	–	–
1,2-Dimethyl-1,4-cyclohexadiene [b]	17351-28-9	1215	–	0.04±0.03 (6)	–	1.06±0.43 (5)
Aldehyde						
Heptanal [a]	111-71-7	1209	35.23±5.73 (7)	1.64±0.93 (5)	12.83±2.62 (8)	1.82±1.15 (5)
(*Z*)-2-Heptenal [b]	57266-86-1	1340	–	0.07±0.03 (11)	–	–
(2*E*,4*E*)-Hepta-2,4-dienal [b]	4313-05-3	1482	–	–	–	0.97±0.39 (6)
2,4-Octadienal [b]	30361-28-5	1580	–	0.01±0.01 (7)	–	–
Ketone						
2-Nonanone [a]	821-55-6	1401	–	4.37±3.31 (4)	–	–
2,5-Hexanedione [b]	110-13-4	1524	–	0.01±0.01 (5)	–	–
2,2,5-Trimethyl-2,6-heptadien-4-one [b]	546-49-6	1584	–	0.02±0.01 (8)	–	–
Dihydro-2(3H)-Furanone [b]	96-48-0	1659	–	–	3.79±1.02 (8)	–
Alcohol						
1-Octen-3-ol [a]	3391-86-4	1461	4.17±0.91 (6)	0.01±0.01 (1)	0.34±0.34 (1)	–
Benzenoids						
Anisole [a]	100-66-3	1357	–	6.75±2.85 (6)	–	14.86±6.61 (4)
Benzaldehyde [a]	100-52-7	1553	50.31±6.02 (7)	0.41±0.13 (14)	63.12±4.86 (8)	2.80±0.60 (11)
Methyl benzoate [a]	93-58-3	1638	–	0.01±0.00 (3)	–	0.01±0.01 (3)
Methyl salicylate [a]	119-36-8	1808	–	tr (3)	–	tr (2)
Benzylalcohol [a]	100-51-6	1896	–	0.02±0.01 (10)	7.48±0.71 (8)	0.08±0.04 (8)
2-Methylphenol [b]	95-48-7	2023	–	0.01±0.00 (5)	–	–
Phenol [a]	108-95-2	2032	3.55±0.72 (6)	0.04±0.01 (13)	2.03±0.30 (8)	0.27±0.09 (11)
Terpenoids						
Monoterpenoids						
α-Pinene [a]	7785-70-8	1095	–	16.29±6.52 (5)	–	12.24±6.46 (3)
β-Pinene [a]	127-91-3	1194	3.55±2.31 (2)	1.52±0.77 (9)	–	1.62±0.97 (3)
β-Myrcene [a]	123-35-3	1199	–	0.04±0.04 (2)	–	0.28±0.28 (3)
Limonene [a]	138-86-3	1224	–	0.28±0.15 (4)	–	0.35±0.23 (3)
cis-β-Ocimene [a]	3338-55-4	1275	–	0.16±0.12 (2)	–	–
p-Cymene [a]	99-87-6	1383	–	0.05±0.02 (8)	–	–
cis-Linalool Oxide (Furanoid) [a]	5989-33-3	1467	–	0.10±0.06 (3)	–	0.13±0.08 3)
Linalool [a]	78-70-6	1562	3.19±0.88 (5)	0.12±0.05 (11)	10.41±2.43 (7)	0.03±0.02 (3)
β-Cyclocitral [b]	432-25-7	1647	–	0.01±0.00 (5)	–	–
Sesquiterpenoid						
β-Caryophyllene [a]	87-44-5	1636	–	tr (6)	–	tr (2)
Nitrogen-containing compound						
2-Methoxy-3-methylpyrazine [c]	2847-30-5	1392	–	0.17±0.07 (5)	–	–

[a] Identifications based on mass spectrum, Kovats retention index and authentic standard.
[b] Identifications based on mass spectrum and Kovats retention index.
[c] Identification based on mass spectrum only. tr=trace amounts (<0.01%).

cones is the major difference between female and male cones before pollination.

During pollen release, the major compounds in male cones were unsaturated hydrocarbons, (3*E*)-1,3-octadiene (54.2%) and (3*E*,5*Z*)-1,3,5-octatriene (13.4%), the monoterpene α-pinene (16.3%), and the benzenoid anisole (6.7%) (Table 1). In receptive female cones, the same major compounds were detected but the ranking was slightly different, comprising (3*E*,5*Z*)-1,3,5-octatriene (47.7%) and (3*E*)-1,3-octadiene (15.8%), anisole (14.9%), and α-pinene (12.2%).

The NMDS analysis of volatile compounds emitted from female cones before and during receptivity, and from male cones before and during pollen shed (Fig. 2), showed a significant separation between life stages and sexes (NMDS stress value 0.06;

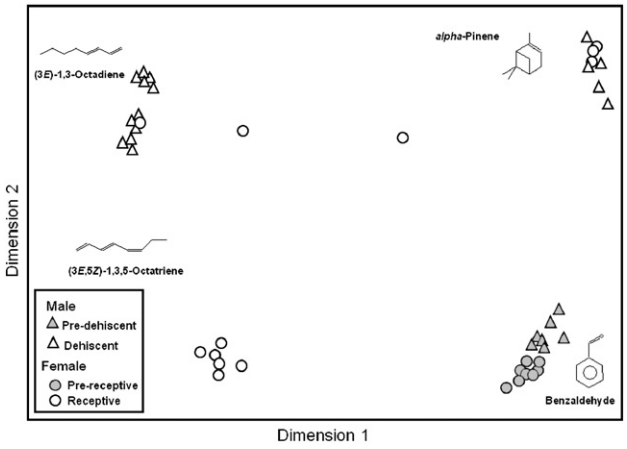

Fig. 2. Non-metric multidimensional scaling (NMDS) based on Bray–Curtis similarities of the odour composition of male and female cones *E. natalensis* before and during pollination. (NMDS 2D stress value = 0.06).

ANOSIM, $R = 0.402$, $P < 0.01$). There was no distinction between pre-receptive female cones and pre-dehiscent male cones, which were characterized by the presence of benzaldehye together with smaller amounts of benzyl alcohol, heptanal, 1-octen-3-ol, dihydro-2(3)-furanone, and linalool. Most receptive female cones formed a cluster which was characterised by the dominance of (3*E*,5*Z*)-1,3,5-octatriene. Male cones formed two clusters, one dominated by (3*E*)-1,3-octadiene and a second by α-pinene, with a few female cones also falling into these clusters (Fig. 2). Other compounds emitted in small relative amounts contributed to the separation of the stages. For example, the compounds (*Z*)-2-heptenal, 2,4-octadienal, 1,2-dimethyl-1,4-cyclohexadiene, 2-nonanone, 2,5-hexanedione, 2,2,5-trimethyl-2,6-heptadien-4-one, 2-methoxy-3-methylpyrazine, and *p*-cymene are emitted only by pollen shedding cones whereas (2*E*,4*E*)-hepta-2-4-dienal is emitted only by receptive female cones.

3.2. Thermogenesis

There was little or no increase in temperature in female cones before and during receptivity. In non-receptive and receptive female cones, cone temperatures never rose above the maximum ambient air temperature, indicating that there was no thermogenesis. There was however a lag in the cooling of female cones as ambient temperatures dropped in the late afternoon resulting in cone temperatures that were consistently above ambient from 1400 h to 1900 h (Fig. 3a). Heating pattern of male cones before pollen shed was similar to that of female cones before receptive phase. Cone temperatures were consistently below ambient temperature from morning to late afternoon (Fig. 3b). In contrast, cone temperature of male cones consistently rose above the maximum concurrent ambient temperature during pollen shed (Fig. 3b), with a peak in the early afternoon (around 1400 h). During pollen shed, daily maximum mean cone temperature ranged between 28.0 and 31.8 °C and exceeded mean ambient temperature by between 10.1 and 11.1 °C.

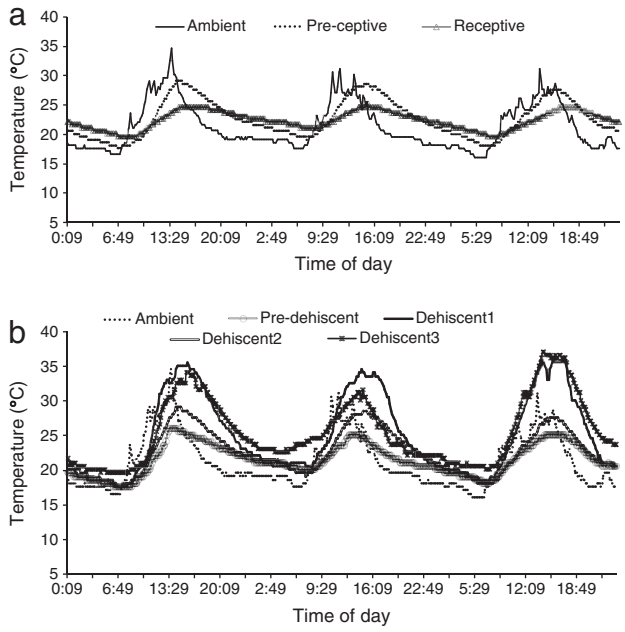

Fig. 3. Pattern of thermogenesis in relation to ambient temperature: (a) female cones (one each) before and during receptivity; (b) male cones before (one example shown) and during pollen shed (three examples shown).

3.3. Insect visitors to male and female cones

Before maturity, when sporophylls were not yet separated, no insects were found on male cones. At pollen shed, three beetle species (Coleoptera) were present in male cones, namely an undescribed Erotylidae, *Metacucujus goodei* Endrödy-Younga (Boganiidae), and an undescribed species of *Porthetes* (Curculionidae) that is probably the same taxon as *Porthetes* sp. 14.2 and 14.3 referred to by Downie et al. (2008) (Fig. 4a–c). Older male cones had beetle larvae in the microsporophylls. Adult *Porthetes* sp. were active on the cone surface while Erotylidae sp. nov. and *M. goodei* were mostly moving in between the sporophylls and along the cone axis. *Porthetes* sp. specimens were most abundant on the male cones (75.71 ± 8.31 individuals; mean ± S.E) and their abundance was significantly greater than *M. goodei* (36.14 ± 2.93) and Erotylidae sp nov. (39.00 ± 7.15) (ANOVA, $F_{2,\ 18} = 6.02$: $P < 0.01$). Sampling of female cones revealed that, prior to receptivity, female and male *Antliarhinus signatus* and *Antliarhinus zamiae* beetles (Fig. 4d–f) were crawling over the surface of the cones. On receptive cones, we also found Erotylidae sp. nov. and *Porthetes* sp., the same taxa as on pollen shedding cones. While *Antliarhinus* spp. could be seen mating and actively crawling over the cone surface, Erotylidae sp. nov. and *Porthetes* sp. were mostly found in between the tightly packed megasporophylls which hampered their collection.

4. Discussion

Our results show that pollen shedding in male *E. natalensis* cones was characterised by a distinct period of thermogenic activity accompanied by volatile emissions that were significantly different from the emissions preceding pollen shed. At

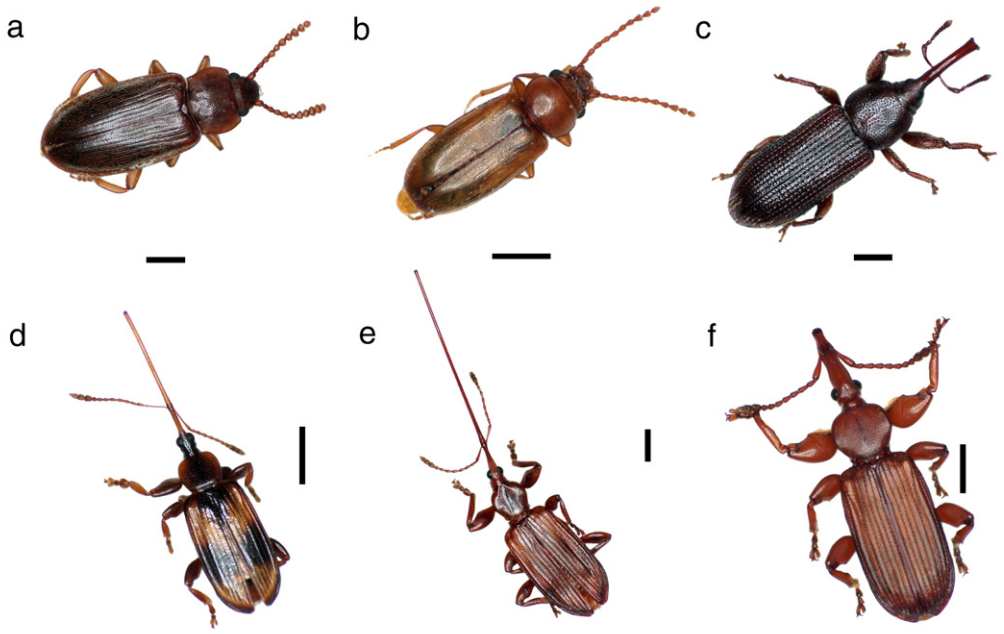

Fig. 4. (a–f) Insects which visit male and/or female cones of *Encephalartos natalensis*. (a) An undescribed Erotylidae sp nov. (male and female cones); (b) *Metacucujus* goodei (male cones); (c) *Porthetes* sp. (male and female cones); (d) female *Antliarhinus signatus* (female cones); (e) female *Antliarhinus zamiae* (female cones); (f) male *Antliarhinus zamiae* (female cones). Scale bars=1000 μm.

least three species of beetles were active on the male cones during pollen shed, all belonging to genera that are involved in the pollination of other *Encephalartos* species. In contrast, female cones did not appear to be thermogenic, but receptive female cones also emitted characteristic volatile profiles that were significantly different to those emitted by pre-receptive cones. The odour constituents of receptive female cones were mostly a subset of the odour constituents of male cones, but the relative amounts of major components differed substantially between sexes. Nevertheless, the beetles occurring on male cones were also present on female cones. This study thus provides new insights into the changes in volatile emissions associated with pollen shedding and receptivity in cycad cones.

Plant scents are made up of a complex blend of compounds that belong to different biosynthetic pathways; they may vary in number of constituents, composition, relative amounts, and in their temporal and spatial emission patterns (Raguso, 2004; Knudsen et al., 2006;). The complexity of these blends is evident in the differences between mature female and male cones. Odours from male cones comprised a greater number of compounds with (3*E*)-1,3-octadiene being the dominant compound followed by (3*E*,5*Z*)-1,3,5-octatriene, whereas odours from female cones comprised a subset of the compounds found in male cones and were characterised by high levels of (3*E*,5*Z*)-1,3,5-octatriene and (3*E*)-1,3-octadiene respectively (Table 1). Generally, it may be expected that there would be some congruence in the cues associated with male and female cones since the pollinator needs to interact with both sexes and other studies have shown that female cones emit similar odours to male cones but at lower concentrations (Terry et al., 2004). In addition to the overall blend of compounds, differences in the concentration of specific compounds can influence pollinator behaviour. In the Australian cycad, *Macrozamia lucida*, low concentrations of *β*-myrcene attracted pollen vectors whereas high concentrations of the same compound repelled them (Terry et al., 2004; Terry et al., 2007). This 'push–pull' interaction has not been confirmed in any other cycads but emphasizes the potential importance of differences in the relative and absolute concentrations of different compounds.

The NMDS analysis indicated that there were differences between samples, based primarily on volatile profiles of mature male cones. Some samples of *E. natalensis* were characterised by unsaturated hydrocarbons, made up of (3*E*)-1,3-octadiene and (3*E,5Z*)-1,3,5-octatriene, whereas other samples were dominated by the monoterpene, α-pinene. In male and female cones, (3*E*)-1,3-octadiene and (3*E,5Z*)-1,3,5-octatriene were associated with 1,2-dimethyl-1,4-cyclohexadiene and (*E,E,E*)-2,4,6-octatriene while α-pinene was associated with anisole, 2-nonanone, and *cis*-linalool oxide. Plants which emitted unsaturated hydrocarbons did not emit the terpenoid and vice versa. The emission patterns of these minor volatiles might reflect different biosynthetic pathways (Knudsen et al., 2006) for the unsaturated hydrocarbons and monoterpenes. The variation in the groupings might also be influenced by geographic origin. All *E. natalensis* sampled from the Pietermaritzburg campus at UKZN emitted α-pinene and anisole as the dominant compounds while those sampled from the KZN National Botanical Garden and conservation area of Hilton College Pietermaritzburg emitted the unsaturated hydrocarbons (3*E*)-1,3-octadiene and (3*E,5Z*)-1,3,5-octatriene. Different forms of *E. natalensis* have been recognised, based on leaf characteristics and the extent of woolly tomentum (Giddy, 1978; Goode, 2001; Grobbelaar, 2002) and the occurrence of different dominant volatiles suggest that a more critical

sampling approach is required to test for differences in volatile profiles between populations of *E. natalensis*, and between garden and natural populations.

With the exception of the unsaturated hydrocarbons, many of the volatiles found in *E. natalensis* are well known floral volatiles occurring in many flowering plants (Knudsen et al., 2006) and may have different biological roles. Cone volatiles have been analysed for only three African cycads species (Pellmyr et al., 1991; Proches and Johnson, 2009; Suinyuy et al., in press) and eight other cycad species (Pellmyr et al., 1991; Terry et al., 2004; Azuma and Kono, 2006) out of an estimated 300 species (Hill et al., 2004). As a result, any comparative analysis of cone volatiles from *E. natalensis* with chemical compounds from other cycads will need to be revised as additional information becomes available.

It is notable that *E. natalensis* shares only one major compound (3*E*)-1,3-octadiene with *E. altensteinii,* which is a morphologically similar and closely related species (Treutlein et al., 2005; Vorster 2004). A number of minor compounds, such as *β*-myrcene, *cis*-*β*-ocimene, limonene, benzyl alcohol, and methyl salicyclate also occur in both *E. natalensis* and *E. altensteinii* and a few other minor compounds are common to *E. natalensis*, *E. altensteinii* and *E. villosus*, e.g. α-pinene, linalool, and benzaldehyde. This study is the first to report the presence of (3*E*,5*Z*)-1,3,5-octatriene in cycads although this compound, together with (3*E*)-1,3-octadiene and anisole have also been recorded from some populations of *E. villosus* from KwaZulu-Natal (Suinyuy, unpublished data). Little is known about the biological function of (3*E*,5*Z*)-1,3,5-octatriene except that it has been reported as a chemo-attractant in marine brown algae (Kajiwara et al., 1980; Boland, 1995). Our identification of this isomer in the scent of *E. natalensis* is tentative and based on mass spectra and the Kovats retention index (Table 1). Roman Kaiser (pers. comm.) has also indicated that this particular isomer tends to be the one most commonly encountered in floral scents.

A large number of monoterpenes were detected in cone volatiles from *E. natalensis* and have been recorded from *E. altensteinii* (Pellmyr et al., 1991), *E. villosus (*Suinyuy et al., in press), as well as species of *Macrozamia* and *Zamia* (Pellmyr et al., 1991; Terry et al., 2004). Despite the prevalence of monoterpenes in cycad volatiles, relatively few have been studied in relation to their function in pollination (e.g. Terry et al., 2007) and this gap should be addressed in future studies. A similar situation exists for benzenoids, which were the second most numerous volatile compounds emitted by *E. natalensis* cones and which have been recorded in headspace samples from many flowering plants (Knudsen et al., 2006). Anisole and phenol have not been previously reported in cycad species, but are also emitted by members of the Araceae (Borg-Karlson et al., 1994; Skubatz et al., 1996). Anisole acts as a sex pheromone in some Coleoptera (Leal et al., 1996) and an attractant for bark beetles (Vrkocova et al., 2000) while phenol is an attractant of *Cyclocephala* sp. (Gruner and Marival, 1974) and an aggregation pheromone of the palm weevil *Rhynchophorus* (Oehlschlager et al., 1995). These compounds could therefore play a role in mediating the behaviour of cycad insects.

Temperature measurements in male cones of *E. natalensis* showed a single peak associated with thermogenesis occurring from early afternoon (1300 h) to late afternoon (1600 h). The cone temperature reached a peak of between 7.0 °C and 11.0 °C above ambient between 1400 h and 1530 h, before it started cooling down. The frequent co-occurrence of heat production and volatile emissions in cycads indicates that this is a syndrome which may regulate insect pollinator behaviour (Tang, 1987b; Seymour et al., 2004; Terry et al., 2004). Studies of other cycads suggest that thermogenesis enhances volatilization of odour, promotes male cone elongation and separation of sporophylls (Tang 1987a,b; Tang et al., 1987; Tang 1993), and may increase growth and survival in pollinating insects (Terry et al., 2004). Seymour and Schultze-Motel (1997) suggest that the heat generated by thermogenesis increases the activity of insect pollinators. In angiosperm systems, thermogenesis may enhance scent production to attract insects, increase insect activity, facilitate dehiscence of pollen sacs (Seymour and Matthews, 2006), and provide warm habitats (Azuma et al., 1999). The daily timing of peak temperatures in male cones differs between cycad species, from late morning to evening (e.g. Donaldson, 1997; Seymour et al., 2004; Suinyuy et al., in press; Tang, 1987b; Terry 2001; Terry et al., 2004) and may represent a species specific signal. The timing of peak heat production in *E. natalensis* was consistently earlier (1400 h–1530 h) than in *E. villosus* (1700 h–1830 h) (Donaldson, 1997; Suinyuy et al., in press) but was similar to some studies of *E. altensteinii* (Tang, 1987a).

The insects observed on pollen shedding cones of *E. natalensis* (Erotylidae nov. sp., *M. goodei*, and *Porthetes* sp.) all belong to genera or families where at least one species has been shown to pollinate other *Encephalartos* spp. (Donaldson, 1997; Donaldson et al., 1995; Suinyuy et al., 2009). The *Porthetes* is morphologically very similar to a species originally collected from *E. altensteinii* (Oberprieler, 1995) but molecular studies (Downie et al., 2008) indicated that similar specimens collected from *E. lebomboensis* male cones and the old leaf bases of *E. natalensis* were distinct from the specimens from *E. altensteinii* (*Porthetes* sp. 14.2, 14.3 in Downie et al., 2008). Our study represents the first record of this *Porthetes* from male and female cones of *E. natalensis*. All insects seen on male cones were covered with pollen so there is reasonable circumstantial evidence that these taxa also play a role in the pollination of *E. natalensis*. At this stage there is no experimental evidence to confirm their role in pollination. Although *M. goodei* was not recorded from female cones, the same species was considered to play some role in the pollination of *E. villosus* (Donaldson, 1997) where another species of *Porthetes* was the main pollinator.

In conclusion, this study forms part of a growing body of work on the pollination biology of cycads, including evidence for volatile emissions and thermogenesis. It is the first study to show thermogenesis in *E. natalensis* and to analyse the volatile odours emitted before and during pollination. It also provides the first report of (3*E*,5*Z*)-1,3,5-octatriene from cycads and includes new records for insects associated with the cones of *E. natalensis.* At present, there is only a circumstantial link between thermogenesis, volatile emissions and pollination in *E. natalensis* and further experiments are required to test how

insects respond to cone temperature and volatiles, as has been done for some other species (e.g. Suinyuy et al., in press; Terry et al., 2007). Nevertheless, identifying the volatile compounds is a first step that needs to be followed by studies to identify which compounds generate physiological responses, e.g. using gas chromatography–electroantennogram detection, and field tests to determine behavioural responses (Schiestl and Marrion-Poll, 2002).

Acknowledgements

The authors thank Dr. Andreas Jürgens for assisting with volatile analysis, Sandy-Lynn Steenhuisen for technical support in the laboratory, and Taina Witt for carefully reading through the manuscript and giving helpful comments. We also thank Ezemvelo KZN Wildlife for issuing permits, John Roff of Hilton College Pietermaritzburg, the staff of the KZN National Botanical Garden in Pietermaritzburg and the horticulturist of UKZN for granting access to plants. This study was financially supported by the South African National Biodiversity Institute, Fairchild Tropical Botanic Garden, University of Cape Town and University of KwaZulu-Natal.

References

Azuma, H., Kono, M., 2006. Estragole (4-allylanisole) is the primary compound in volatiles emitted from male and female cones of *Cycas revoluta*. Journal of Plant Research 119, 671–676.

Azuma, H., Thien, L.B., Kawano, S., 1999. Floral scent, leaf volatiles and thermogenic flowers in Magnoliaceae. Plant Species Biology 14, 121–127.

Boland, W., 1995. The chemistry of gamete attraction: chemical structures, biosynthesis, and (a)biotic degradation of algal pheromones. Proceedings of the National Academy of Sciences of the United States of America 92, 37–43.

Borg-Karlson, A.-K., Englund, F.O., Unelius, C.R., 1994. Dimethyl oligosulphides, major volatiles released from *Sauromatum guttatum* and *Phallus impudicus*. Phytochemistry 35, 321–323.

Bronstein, J.L., Alarcon, R., Geber, M., 2006. The evolution of plant–insect mutualisms. The New Phytologist 172, 412–428.

Chamberlain, J., 1935. Gymnosperm Structure and Evolution. University of Chicago Press, Chicago, IL, USA.

Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology 18, 117–143.

Clarke, K.R., Gorley, R.N., 2006. Primer v6: User Manual/Tutorial. Primer-E, Plymouth.

Donaldson, J.S., 1997. Is there a floral parasite mutualism in cycad pollination? The pollination biology of *Encephalartos villosus* (Zamiaceae). American Journal of Botany 84, 1398–1406.

Donaldson, J.S., Nänni, I., Bosenberg, J.D., 1995. The role of insects in the pollination of *Encephalartos cycadifolius*. In: Vorster, P. (Ed.), Proceedings of the Third International Conference on Cycad Biology. The Cycad Society of South Africa, Stellenbosch, pp. 423–434.

Downie, D.A., Donaldson, J.S., Oberprieler, R.G., 2008. Molecular systematics and evolution in an African cycad–weevil interaction: Amorphocerini (Coleoptera: Curculionidae: Molytinae) weevils on *Encephalartos*. Molecular Phylogenetics and Evolution 47, 102–116.

Giddy, C., 1978. Cycads of South Africa. Struik Publishers, Cape Town.

Goode, D., 2001. Cycads of Africa. D&E Cycads of Africa Publishers.

Grobbelaar, N., 2002. Cycads with Special Reference to the South African Species. Four images Bureau & Printers, Pretoria, South Africa.

Gruner, L., Marival, D., 1974. The attraction of males of the West Indian beetle *Cyclocephala insulicola* Arrow by phenol (Coleoptera:Dynastidae). Comptes-rendus des Seances de l'Académie d'Agriculture de France 60, 203–208.

Hall, J.A., Walter, G.H., Bergstrom, D.M., Machin, P., 2004. Pollination ecology of the Australian cycad *Lepidozamia peroffskyana* (Zamiaceae). Australian Journal of Botany 52, 333–343.

Hill, K.D., Stevenson, D.W., Osborne, R., 2004. The world list of cycads. Botanical Review 70, 274–298.

Jacot-Guillarmod, A., 1958. Temperature variations in male cones of *Encephalartos*. Nature 182, 474.

Jones, D.L., 1993. Cycads of the World. Ancient Plants of Today's Landscape, First edition. Reed, Chatswood, Australia.

Jürgens, A., 2009. The hidden language of flowering plants: floral odours as a key for understanding angiosperm evolution? The New Phytologist 183, 240–243.

Kajiwara, T., Kadoma, K., Hatanaka, A., 1980. Attractions of male gamete from marine brown alga *Sargassum horneri*. Bulletin of the Japanese Society of Scientific Fisheries 46, 555–557.

Knudsen, J.T., Eriksson, R., Gershenzon, J., Ståhl, B., 2006. Diversity and distribution of floral scent. Botanical Review 72, 1–120.

Kono, M., Tobe, H., 2007. Is *Cycas revoluta* (Cycadaceae) wind- or insect-pollinated? American Journal of Botany 94, 847–855.

Leal, W.S., Yadava, C.P.S., Vijayvergia, J.N., 1996. Aggregation of the scarab beetle *Holotrichia consanguinea* in response to female released pheromone suggests secondary function hypothesis for semiochemical. Journal of Chemical Ecology 22, 1557–1566.

Niklas, K.J., Norstog, K.J., 1984. Aerodynamics and pollen grain depositional patterns on cycad megastrobili: implications on the reproduction of three Cycad Genera (*Cycas*, *Dioon*, and *Zamia*). Botanical Gazette 145, 92–104.

Norstog, K.J., Nicholls, T.J., 1997. The Biology of the Cycads. Cornell University Press, Ithaca.

Norstog, K.J., Stevenson, D.W., Niklas, K.J., 1986. The role of beetles in the pollination of *Zamia furfuracea* L. fil. (Zamiaceae). Biotropica 18, 300–306.

Oberprieler, R.G., 1995. The weevils (Coleoptera: Curculionoidea) associated with cycads. 1. Classification, relationships, and biology. In: Vorster, P. (Ed.), Proceedings of the Third International Conference of Cycad Biology. Cycad Society of South Africa, Stellenbosch, South Africa, pp. 295–334.

Oberprieler, R.G., 2004. "Evil weevils" — the key to cycad survival and diversification? In: Lindstrom, A. (Ed.), Proceedings of the 6th International Cycad Conference on Cycad Biology. Nong Nooch Tropical Botanical Garden, Thailand, pp. 170–194.

Oehlschlager, A.C., Prior, R.N.B., Perez, A.L., Gries, R., Gries, G., Pierce Jr., H.D., Laup, S., 1995. Structure, chirality, and field testing of a male-produced aggregation pheromone of Asian palm weevil *Rhynchophorus bilineatus* (Montr.) (Coleoptera: Curculionidae). Journal of Chemical Ecology 21, 1619–1629.

Pearson, H.H.W., 1906. Notes on South African cycads. Transactions of the South African Philosophical Society 16, 341–354.

Pellmyr, O., 1992. Evolution of insect pollination and angiosperm diversification. Trends in Ecology & Evolution 7, 46–49.

Pellmyr, O., Thien, L.B., 1986. Insect reproduction and floral fragrances: keys to the evolution of angiosperms. Taxon 35, 76–85.

Pellmyr, O., Tang, W., Groth, I., Bergström, G., Thien, L.B., 1991. Cycad cone and angiosperm floral volatiles: inferences for the evolution of insect pollination. Biochemical Systematics and Ecology 19, 623–627.

Proches, S., Johnson, S.D., 2009. Beetle pollination of the fruit-scented cones of the South African cycad S*tangeria eriopus*. American Journal of Botany 96, 1722–1730.

Raguso, R.A., 2004. Why do flowers smell? The chemical ecology of fragrance-driven pollinator. In: Cardé, R.T., Millar, J.G. (Eds.), Advances in Insect Chemical Ecology. Cambridge University Press, Cambridge, UK, pp. 141–178.

Rattray, G., 1913. Notes on the pollination of some South African cycads. Transactions of the Royal Society of South Africa 3, 259–270.

Schiestl, F.P., Marrion-Poll, F., 2002. Detection of physiologically active flower volatiles using gas chromatography coupled with electroantennography. In: Jackson, J.F., Linskens, H.F., Inman, R. (Eds.), Molecular Methods of Plant Analysis. : Analysis of Taste and Aroma, 21. Springer, Berlin, pp. 173–198.

Seymour, R.S., Matthews, P.D.G., 2006. The role of themogenesis in the pollination biology of the Amazon waterlilly *Victoria amazonica*. Annals of Botany 98, 1129–1135.

Seymour, R.S., Schultze-Motel, P., 1997. Heat producing flowers. Endeavour 21, 125–129.

Seymour, R.S., Terry, I., Roemer, R.B., 2004. Respiration and thermogenesis by cones of the Australian cycad *Macrozamia machinii*. Functional Ecology 18, 925–930.

Skubatz, H., Kunkel, D.D., Howald, W.N., Trenkle, R., Mookherjee, B., 1996. The *Sauromatum guttatum* appendix as an osmophore: excretory pathways, composition of volatiles and attractiveness to insects. The New Phytologist 134, 631–640.

Stevenson, D.W., Norstog, K.J., Fawcett, P.K.S., 1998. Pollination biology of cycads. In: Owens, S.J., Rudall, P.J. (Eds.), Reproductive Biology. Royal Botanic Gardens, Kew, pp. 277–294.

Suinyuy, T.N., Donaldson, J.S., Johnson, S.D., 2009. Insect pollination in the African cycad *Encephalartos friderici-guilielmi* Lehm. South African Journal of Botany 75, 682–688.

Suinyuy, T.N., Donaldson, J.S., Johnson, S.D., Bösenberg, J.D. (in press). Role of cycad cone volatile emissions and thermogenesis in the pollination of *Encephalartos villosus* Lem.: preliminary findings from studies of plant traits and insect responses. Proceedings of the 8th International Conference of Cycad Biology, 2008, Panama City, Panama. New York Botanic Garden Press.

Tang, W., 1987a. Heat production in cycad cones. Botanical Gazette 148, 165–174.

Tang, W., 1987b. Insect pollination in the cycad *Zamia pumila* (Zamiaceae). American Journal of Botany 74, 90–99.

Tang, W., 1993. Heat and odour production in cycad cones and their role in insect pollination. In: Stevenson, D.W., Norstog, K.J. (Eds.), Proceedings of the Second International Conference of Cycad Biology. Palm and Cycad Societies of Australia, Milton Queensland, Australia, pp. 140–147.

Tang, W., Sternberg, L., Price, D., 1987. Metabolic aspects of thermogenesis in male cones of five cycad species. American Journal of Botany 74, 1555–1559.

Terry, L.I., 2001. Thrips and weevils as dual, specialist pollinators of the Australian cycad *Macrozamia communis* (Zamiaceae). International Journal of Plant Sciences 162, 1293–1305.

Terry, I., Moore, C.J., Walter, G.H., Forster, P.I., Roemer, R.B., Donaldson, J.S., Machin, P.J., 2004. Association of cone thermogenesis and volatiles with pollinator specificity in *Macrozamia* cycads. Plant Systematics and Evolution 243, 233–247.

Terry, L.I., Walter, G.H., Donaldson, J.S., Snow, E., Forster, P.I., Machin, P.J., 2005. Pollination of Australian *Macrozamia* cycads: effectiveness and behaviour of specialist vectors in a dependent mutualism. American Journal of Botany 92, 116–125.

Terry, I., Walter, G.H., Moore, C., Roemer, R., Hull, C., 2007. Odour-mediated push–pull pollination in cycads. Science 318, 70.

Tholl, D., Röse, U.R.S., 2006. Detection and identification of floral scent compounds. In: Dudareva, N., Pichersky, E. (Eds.), Biology of Floral Scent. CRC Press, Taylor & Francis Group, New York, USA, pp. 3–25.

Treutlein, J., Vorster, P., Wink, M., 2005. Molecular relationships in *Encephalartos* (Zamiaceae, Cycadales) based on nucleotide sequences of nuclear ITS1 and 2, Rbcl, and genomic ISSR fingerprinting. Plant Biology 7, 79–90.

Vorster, P., 1995. Aspects of the reproduction of cycads. 1. Pollinating mechanisms and the association of Amorphocerus (Curculionidae) with *Encephalartos*. In: Vorster, P. (Ed.), Proceedings of the Third International Conference of Cycad Biology. Cycad Society of South Africa, Stellenbosch, South Africa, pp. 367–378.

Vorster, P., 2004. Classification concepts in *Encephalartos* (Zamiaceae). In: Walters, T., Osborne, R. (Eds.), Cycad Classification Concepts and Recommendations. CABI Publishing, Wallingford, UK, pp. 69–83.

Vovides, P., 1991. Insect symbionts of some Mexican cycads in their natural habitat. Biotropica 23, 102–104.

Vovides, A.P., Ogata, N., Sosa, V., Pena-Garcia, E., 1997. Pollination of endangered Cuban cycad *Microcycas calocoma* (Miq.) A. DC. Botanical Journal of the Linnean Society 125, 201–210.

Vrkocova, P., Valterova, I., Vrkoc, J., Koutek, B., 2000. Volatiles released from oak, a host tree for the bark beetle *Scolytus intricatus*. Biochemical Systematics and Ecology 28, 933–947.

Wilson, G.W., 2002. Insect pollination in the cycad genus *Bowenia* Hook. *Ex* Hook. F. (Stangeriaceae). Biotropica 34, 438–441.

Edited by J Van Staden